

AD_____

AWARD NUMBER: W81XWH-05-1-0173

TITLE: Tissue Microarray Assessment of Novel Prostate Cancer Biomarkers AMACR and EZH2 and Immunologic Response to Them in African-American and Caucasian Men

PRINCIPAL INVESTIGATOR: Rohit Mehra

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: April 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-04-2006		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 7 Mar 2005 – 6 Mar 2006	
4. TITLE AND SUBTITLE Tissue Microarray Assessment of Novel Prostate Cancer Biomarkers AMACR and EZH2 and Immunologic Response to Them in African-American and Caucasian Men				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0173	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rohit Mehra E-Mail: mrohit@med.umich.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Michigan Ann Arbor, Michigan 48109-1274				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Prostate cancer is a leading cause of cancer-related death in American men. Prostate cancer, like other cancers is characterized by complex molecular events influenced by diverse genetic and environmental factors. The objective of the present study was to compare the expression of AMACR and EZH2 in African-American patients and Caucasian patients with prostate cancer and understand their role. We constructed 5 tissue microarrays representing 40 African-American and 159 Caucasian prostate cancer patients and performed immunohistochemistry on these arrays using antibodies to AMACR and EZH2. Protein expression was scored on these arrays for both AMACR and EZH2. We are presently analyzing the data generated from these experiments and looking into the relative levels of the two markers in prostate cancer patients from the two racial subgroups and also for any associations with survival patterns and clinico-pathologic parameters.					
15. SUBJECT TERMS PROSTATE CANCER, ASSOCIATION STUDIES, TUMOR MARKERS, TUMOR IMMUNOLOGY, AMACR					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)
			UU	13	

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	12
Appendices.....	15

Introduction

Background

Prostate cancer is a highly prevalent disease of the Western world (1). Although effective surgical and radiation treatments exist for clinically localized prostate cancer, metastatic prostate cancer remains essentially incurable. A significantly higher mortality rate for prostate cancer in Caucasian than in the African-American population (2) indicates an inherently aggressive biological nature of the malignancy in the latter. Yet, no conclusive data have emerged to date regarding the natural difference in the biology of prostate cancer in the two racial groups (3, 4). Exploring the molecular circuitry that differentiates indolent prostate cancer from the more aggressive ones in African-American population may lead to the identification of new pathways that govern the clinical outcome. Likewise, biomarkers which separate indolent and aggressive prostate cancer will be of immense clinical utility. With the emergence of global profiling strategies, a systematic analysis of genes involved in prostate cancer is now possible.

In our previous study (5), we reported gene expression profiles of benign prostate, clinically localized prostate cancer, and hormone-refractory metastatic prostate cancer. We discovered a cohort of genes which specifically mark the molecular transition from organ confined prostate cancer to metastatic prostate cancer. We recently characterized one of these genes, the Polycomb group (PcG) protein Enhancer of Zeste Homolog 2 (EZH2) (6). Likewise, we showed AMACR (α -methylacyl-CoA racemase) to be overexpressed in prostate cancer at the transcript level by reverse transcriptase polymerase chain reaction (RT-PCR) and at the protein level by immunoblot and immunohistochemical analysis (7).

AMACR in Prostate Cancer

AMACR is a well-characterized enzyme(8) that plays a key role in peroxisomal β -oxidation of dietary branched-chain fatty acids and C27-bile acid intermediates. It catalyzes the conversion of (R)- α -methyl-branched-chain fatty acyl-CoA esters to their (S)-stereoisomers. Only the (S)-stereoisomers can serve as substrates for branched-chain acyl-CoA oxidase during their subsequent peroxisomal β -oxidation. Two aspects of this pathway may have particular relevance for prostate carcinogenesis: (a) the main sources of branched chain fatty acids in humans (milk, beef, and dairy products) , have been implicated as dietary risk factors for prostate cancer (9); and (b) peroxisomal β -oxidation generates hydrogen peroxide (10) , a potential source of procarcinogenic oxidative damage (11).

We reported previously (7) the utility of this marker in the detection of prostate adenocarcinoma, even in very small clinical samples obtained via trans-rectal prostate biopsies. This was done via corroboration of increasing expression in immunohistochemical analysis (IHC) showing an increasing trend in AMACR expression from prostatic intraepithelial neoplasia (PIN) to prostate cancer. Additionally, in this analysis, we quantified the sensitivity and specificity of prostate adenocarcinoma detection via

AMACR staining at 97% and 100% respectively. More recently, our group showed that lower AMACR tissue expression was associated with worse prostate cancer outcome, independent of clinical variables (12). The AMACR cut-point developed using prostate cancer-specific death as the end point predicted PSA failures independent of Gleason score, PSA, and margin status. This is the first study to show that AMACR expression is significantly associated with prostate cancer progression.

EZH2 in Prostate Cancer

Mis-expression of Polycomb group (PcG) proteins can lead to defects in proliferation and tumorigenesis. Our previous studies have shown that dysregulated expression of EZH2 may be involved in the progression of prostate cancer as well as serves as a marker that distinguishes indolent cancer from those at the risk of lethal progression(6).

Our aim in the proposal was (1) *To construct tissue microarray blocks for cohort of African-American men and Caucasian men, for this and future studies* (2) *To delineate if the expression of novel prostate cancer biomarkers, AMACR and EZH2, is different in African-American versus Caucasian men and correlates with clinical outcome* (3) *To determine if the level of immunologic response to AMACR and EZH2 differs in African-American versus Caucasian men and correlates with clinical outcome.*

Research Progress

Tissue Microarray construction

Clinical specimens were obtained from the radical prostatectomy series at the University of Michigan to examine the widest range of the prostate cancer specimens. Prostatectomy cases for the tissue micro-arrays were selected from a cohort of patients who underwent radical retropubic prostatectomy at the University of Michigan for clinically localized prostate cancer. Consecutive cases were taken to ensure clinical follow-up. Clinical and pathologic data for all patients were acquired with approval from the Institutional Review Board at the University of Michigan. Detailed clinical, Pathologic and TMA data are maintained on a secure database. Tumors were staged using the TNM system, and graded using the Gleason grading system. As preparation for construction of the TMAs, all glass slides were reexamined to identify areas of benign prostate, high-grade PIN, and prostate cancer. To optimize the transfer of these designated tissues to the arrays, the area of tumor involvement was encircled on the glass slide as closely around each lesion as possible.

Five TMAs are being used for this study. The TMAs were constructed using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD) following a previously described technique (13, 14). Replicate tissue cores were sampled from the selected tissue types which included benign, prostate intraepithelial neoplasia (PIN) and prostate cancer. The 0.6-mm diameter cores were placed 0.8mm from core center to core center. After construction, 5- μ m sections were cut and hematoxylin-eosin stained to verify the diagnosis.

Representative distribution of African- American and Caucasian patients in the Tissue Microarrays

Benign, PIN and prostate cancer tissues from 40 unique African- American patients and 159 unique Caucasian patients were represented on the 5 TMAs. Benign areas from 116 cases and prostate intraepithelial neoplasia (PIN) areas from 109 cases were plotted on the array.

TMA	Total cases	Benign	PIN	PCA cases	AA cases	Caucasian cases	Other races	Unknown	Caucasian GS3+3	Caucasian GS>3+3	AA GS3+3	AA GS>3+3
1	54	52	49	54	18	36	0	0	16	19	5	13
2	36	36	33	36	12	24	0	0	12	12	7	5
3	30	28	27	30	10	20	0	0	5	14	1	9
4	55	0	0	54	0	42	0	12	17	22	0	0
5	43	0	0	42	0	37	0	7	16	19	0	0
	218	116	109	216	40	159	0	19	66	86	13	27

Table1. Representative distribution of tissues from African-American (AA) and Caucasian patients on the 5 tissue microarrays. There is an even distribution of cases with Gleason score (GS) 3+3 and more than 3+3 in both the racial subgroups. Benign and PIN samples from the prostate cancer cases were also plotted on the array.

Immunohistochemistry and Evaluation for AMACR expression

Standard avidin–biotin complex immunohistochemistry was used. Antibody concentration was optimized to obtain the strongest target staining without background staining. Monoclonal antibody P504S (Zeta Corp., Sierra Madre, CA, USA) was utilized. Following paraffin removal and hydration, the slides were treated with 0.1 mol/l citrate at pH 6.0 in a pressure cooker and microwaved (15 min on high for P504S and 10 min on high for p-AMACR) for optimal antigen retrieval before immunostaining. Staining was performed on an autostainer (Dako Cytomation, Carpinteria, CA, USA). Sections were incubated sequentially with the primary antibody (1 : 40 dilution P504S for 2 h at room temperature/ 1 : 5000 dilution p-AMACR for 30 min at room temperature). The Dako Envision Plus detection system was utilized for P504S localization according to the vendor's protocol. Sections were later washed and treated with diaminobenzidine and hydrogen peroxide for 5 min. Sections were counterstained with hematoxylin. Positive staining of AMACR was identified as cytoplasmic and/or luminal/subluminal granular staining within epithelial cells.

Immunohistochemistry evaluation was carried out with ChromaVision ACIS II version (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA (15). The ACIS uses preprogrammed advanced color detection software that measures immunohistochemical stains intensity (range, 0–255) and percentage expression (0–100%). All of the images were reviewed to distinguish the tumor from benign areas. Tissue area of interest was electronically circled on the computer screen, and only those areas were used to measure

the percentage of the circled cells that stained positive for AMACR (0–100%). The final data were recorded in a Microsoft Excel data sheet and were used for statistical analysis.

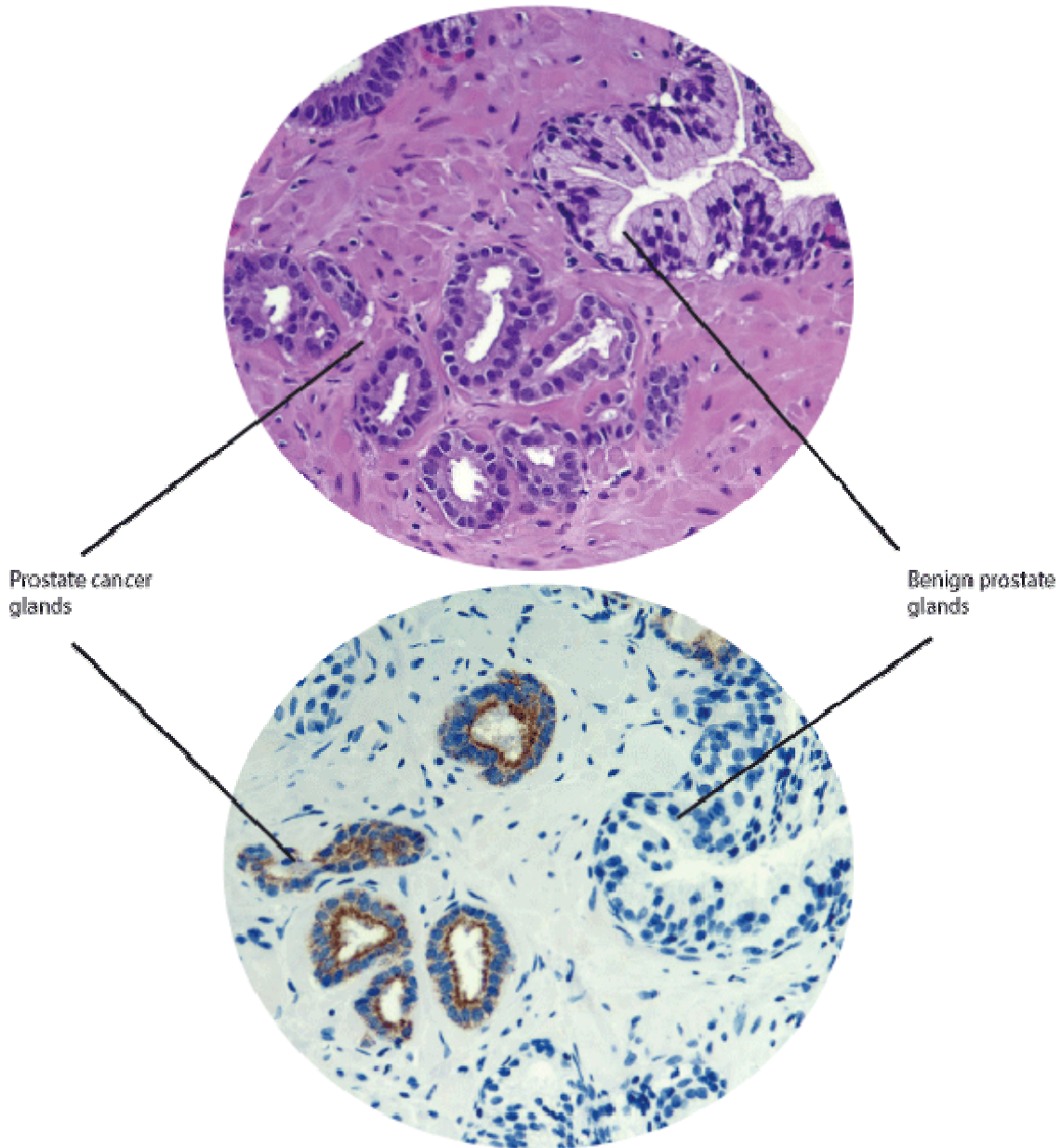
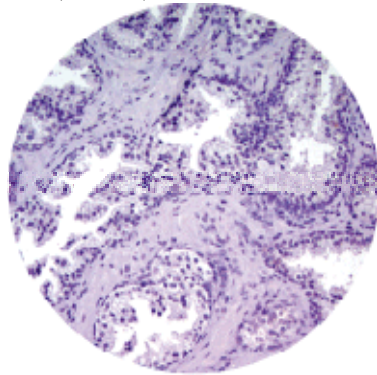


Figure1. Hematoxylin and Eosin staining of benign and prostate cancer glands in the upper image. In the lower image, immunohistochemistry of the same case shows cytoplasmic/ luminal expression of AMACR in the prostate cancer glands preferentially, while the benign glands are negative. Original magnification x100

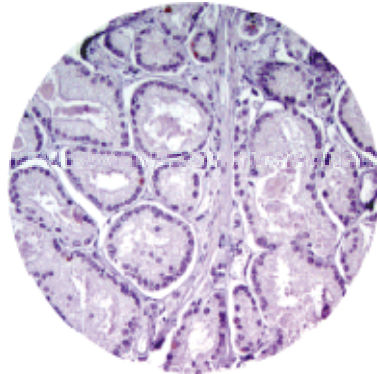
Immunohistochemistry and Evaluation for EZH2 expression

Immunohistochemistry was performed on the tissue microarrays by using standard biotin-avidin complex technique and a rabbit polyclonal antibody against EZH2 (Kind gift from. Prof. Otte). Five- μ m thick paraffin embedded TMAs were de-waxed and hydrated in xylene and ethanol respectively. Antigen retrieval was performed in citrate buffer in pressure cooker at pH 6.0 for 15 minutes. The primary antibody was added to the TMA at a 1:100 dilution. The TMA was then treated with a horseradish peroxidase-labeled secondary antibody for 30 minutes, followed by peroxide/diaminobenzidine substrate/chromagen. The slides were counterstained with hematoxylin. Ezh2 expression was observed in the nucleus, as reported previously (6, 16). Protein expression was scored as negative (score=1), weak (2), moderate (3) and strong (4) in a blinded manner using a validated web- based tool (17, 18).

Benign Prostate



Low Grade Prostate Cancer



High Grade Prostate Cancer

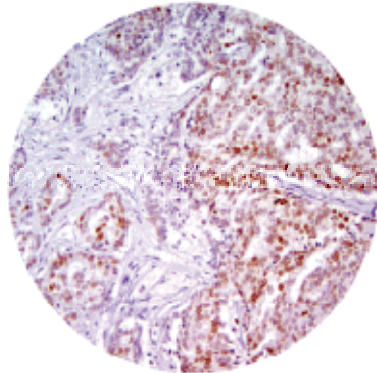


Figure2. Representative tissue elements stained with antibody to EZH2. Immunohistochemical evaluation shows absent nuclear staining in benign prostate, absent or weak staining in a low percent of cells in low grade prostate cancer, and strong expression in high grade prostate cancer. Original magnification x100

Future goals

AMACR and EZH2 are versatile proteins involved in multiple biological pathways. Earlier it has been shown that their expression is upregulated in prostate cancer. We are analyzing the data generated from the immunohistochemical evaluation to compare the expression pattern of the two proteins in Caucasian patients and African- American patients. Our future studies will analyze comprehensively the association of these markers with clinico-pathological parameters in African-American patients and Caucasian patients with prostate cancer. We will also perform univariate and multivariate Cox proportional hazards model of statistically significant covariates to find the link between AMACR and EZH2 expression and prostate cancer recurrence in the two racial groups. Further, we will look for the differences between the immunologic responses to the two biomarkers in the prostate cancer patients from the two groups. We have recently discovered recurrent gene fusions of the 5' untranslated region of TMPRSS2 to ERG or ETV1 in prostate cancer tissues with outlier expression (19). *We will initiate the study to compare the incidence of these gene fusions in the African-American and the Caucasian patients with prostate cancer, and also evaluate any association of these gene fusions with outcome and clinico-pathological parameters in the two racial sub-groups. Fluorescent in situ hybridization will be the principle tool for these experiments.*

Key research accomplishments

1. We have constructed 5 tissue microarrays representing spectrum of prostate pathology including benign glands, prostate intraepithelial neoplasia (PIN) and prostate cancer from 40 African-American and 159 Caucasian patients with prostate cancer.
2. We have stained all these arrays with Hematoxylin and Eosin; and have performed immunohistochemical staining using antibodies to AMACR and EZH2.
3. We have finished evaluation of AMACR and EZH2 protein expression on these tissue microarrays.
4. Biostatistics analysis is underway to compare expression of AMACR and EZH2 in the African-American and Caucasian patients, to look for association with clinico-pathologic parameters and with outcome in the two racial groups.

Reportable Outcomes

1. We have discovered and published the role of recurrent chromosomal arrangements involving ETS family members in prostate cancer(19) Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X. W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. **Science**, 310: 644-648, 2005.
2. We have demonstrated in a recent publication that autoantibodies against peptides derived from prostate-cancer tissue could be used as the basis for a screening test for prostate cancer(20). Wang, X., Yu, J., Sreekumar, A., Varambally, S., Shen, R., Giacherio, D., Mehra, R., Montie, J. E., Pienta, K. J., Sanda, M. G., Kantoff, P. W., Rubin, M. A., Wei, J. T., Ghosh, D., and Chinnaiyan, A. M. Autoantibody signatures in prostate cancer. **N Engl J Med**, 353: 1224-1235, 2005.
3. We have discovered that differential proteomic alterations between metastatic and clinically localized prostate cancer that mapped concordantly to gene transcripts served as predictors of clinical outcome in prostate cancer (21) Varambally, S., Yu, J., Laxman, B., Rhodes, D. R., Mehra, R., Tomlins, S. A., Shah, R. B., Chandran, U., Monzon, F. A., Becich, M. J., Wei, J. T., Pienta, K. J., Ghosh, D., Rubin, M. A., and Chinnaiyan, A. M. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. **Cancer Cell**, 8: 393-406, 2005.
4. We have reported that patients with invasive breast cancer whose tumors expressed low GATA3 had significantly shorter overall and disease-free survival when compared with those whose tumors had high GATA3 levels (22) Mehra, R., Varambally, S., Ding, L., Shen, R., Sabel, M. S., Ghosh, D., Chinnaiyan, A. M., and Kleer, C. G. Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis. **Cancer Res**, 65: 11259-11264, 2005
5. We have developed a new technique, Whole Transcriptome Amplification to profile and archive cDNA from minute tumor samples, this procedure is compatible with partially degraded RNA Tomlins, S. A., Mehra, R., Rhodes, D. R., Shah, R. B., Rubin, M. A., Bruening, E. E., Makarov, V., Chinnaiyan, A. M. Whole transcriptome amplification for gene expression profiling and development of molecular archives. **Neoplasia**, 8: 153-162, 2006
6. We identified a third molecular sub-type of prostate cancer characterized by fusion of TMPRSS2 and ETV4 loci Tomlins, S. A., Mehra, R., Rhodes, D. R., Smith, L. R., Roulston, D., Helgeson, B. E., Cao, X., Wei, J. T., Rubin, M. A., Shah, R. B., Chinnaiyan, A. M. TMPRSS2:ETV4 Gene Fusions Define a Third Molecular Subtype of Prostate Cancer. **Cancer Res**, 66: 3396-3400, 2006

7. We have generated 5 tissue microarrays representing spectrum of prostate pathology, with benign, PIN and prostate cancer samples from 218 patients. These tissue microarrays will be used in the future for detection of proteins by immunohistochemistry; fluorescent in-situ hybridization; RNA in situ hybridization; in situ mi RNA detection, etc.

Conclusion

We have identified a cohort of African- American and Caucasian patients who underwent radical prostatectomy for prostate cancer at the University of Michigan. Five tissue microarrays were constructed from tissues representing 40 African-American and 159 Caucasian patients. AMACR and EZH2 protein expression was explored on these tissue-microarrays using immunohistochemistry. The tissue- microarrays were stained using antibodies to AMACR and EZH2. The tissue microarrays were assessed for the intensity of AMACR and EZH2 expression and the percentage of the cells with that intensity using previously validated methods. Biostatistics analysis is underway to compare AMACR and EZH2 levels in African-American and Caucasian patients, and any association with clinico-pathologic parameters or outcome.

References

1. J. M. Chan, R. M. Jou, P. R. Carroll, *J Urol* **172**, S13 (Nov, 2004).
2. S. L. Parker, K. J. Davis, P. A. Wingo, L. A. Ries, C. W. Heath, Jr., *CA Cancer J Clin* **48**, 31 (Jan-Feb, 1998).
3. I. J. Powell, K. Schwartz, M. Hussain, *Urology* **46**, 825 (Dec, 1995).
4. E. A. Platz, E. B. Rimm, W. C. Willett, P. W. Kantoff, E. Giovannucci, *J Natl Cancer Inst* **92**, 2009 (Dec 20, 2000).
5. S. M. Dhanasekaran *et al.*, *Nature* **412**, 822 (Aug 23, 2001).
6. S. Varambally *et al.*, *Nature* **419**, 624 (Oct 10, 2002).
7. M. A. Rubin *et al.*, *Jama* **287**, 1662 (Apr 3, 2002).
8. S. Ferdinandusse *et al.*, *J Lipid Res* **41**, 1890 (Nov, 2000).
9. J. M. Chan *et al.*, *Am J Clin Nutr* **74**, 549 (Oct, 2001).
10. R. J. Wanders *et al.*, *Biochem Soc Trans* **29**, 250 (May, 2001).
11. T. Tamatani *et al.*, *Int J Oncol* **15**, 743 (Oct, 1999).
12. M. A. Rubin *et al.*, *Cancer Epidemiol Biomarkers Prev* **14**, 1424 (Jun, 2005).
13. J. Kononen *et al.*, *Nat Med* **4**, 844 (Jul, 1998).
14. E. E. Perrone *et al.*, *J Natl Cancer Inst* **92**, 937 (Jun 7, 2000).
15. W. Hilbe *et al.*, *Oncol Rep* **10**, 15 (Jan-Feb, 2003).
16. F. M. Raaphorst *et al.*, *Am J Pathol* **157**, 709 (Sep, 2000).
17. S. Manley, N. R. Mucci, A. M. De Marzo, M. A. Rubin, *Am J Pathol* **159**, 837 (Sep, 2001).
18. G. S. Bova *et al.*, *Hum Pathol* **32**, 417 (Apr, 2001).
19. S. A. Tomlins *et al.*, *Science* **310**, 644 (Oct 28, 2005).
20. X. Wang *et al.*, *N Engl J Med* **353**, 1224 (Sep 22, 2005).
21. S. Varambally *et al.*, *Cancer Cell* **8**, 393 (Nov, 2005).
22. R. Mehra *et al.*, *Cancer Res* **65**, 11259 (Dec 15, 2005).

Appendices

Abbreviations

AMACR	α -methyacyl-CoA racemase
EZH2	Enhancer of Zeste Homolog 2
IHC	Immunohistochemistry
PCA	Prostate Cancer
PcG	Polycomb group proteins
PIN	Prostatic intraepithelial neoplasia
RT-PCR	Reverse transcriptase polymerase chain reaction
TMA	Tissue Microarray
TNM	Primary tumour (T), Regional lymph nodes (N), Distant metastasis (M)